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Kallikrein-related peptidases protein expression in lymphoid tissues suggests potential implications in immune response

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Running title: KLK protein expression in lymphoid tissues

Abstract

Objectives: Kallikrein-related peptidases (KLKs) are a subgroup of 15 secreted chymotrypsin- and trypsin-like serine proteases that have been reported to possess novel functions in innate immunity and inflammation. Since the potential role of KLKs in immunity has not been studied in detail at the protein level, we examined the expression pattern of 12 members of the KLK family in immune-related tissues.

Design & Methods : Protein expression in tissue extracts was evaluated using immunoassays (ELISA). Immunohistochemistry (IHC) was performed on representative sections of tonsil and lymph nodes to determine the cellular localization of the KLK family members.

Results: ELISA profiling of KLK3-KLK15 (except KLK12) revealed higher protein levels in the tonsil, compared to the lymph nodes and spleen. Relatively high protein levels in the tonsil were observed for KLK7, KLK9, KLK10 and KLK13. Expression of these KLKs was significantly lower in lymph nodes and spleen. IHC analysis in tonsil unveiled that KLK9 and KLK10 were differentially expressed in lymphoid cells. KLK9 was strongly expressed in the germinal center of lymphoid follicles where activated B-cells reside, whereas KLK10 was expressed in the follicular dendritic cells (FDCs) that are vital for maintaining the cycle of B cell maturation.

Conclusion: Overall, our study revealed the possible implications of KLK expression and regulation in the immune cells of lymphoid tissues.

Keywords

kallikreins; immunohistochemistry; lymphoid tissues; tonsil; immune system; immunoassays

1. Introduction

Proteolysis plays a central role in immune functions, such as immune cell activation, inflammatory cell extravasation, cytokine production and complement activation [1, 2]. A wide variety of serine proteases have been discovered in immune cells, such as cytotoxic lymphocytes and neutrophils [1]. Serine, cysteine, and metalloproteases have also been associated with several immune-related tissues [3]. Proteases can also regulate other proteolytic enzymes [4], with the deregulation of protease networks being linked to several pathologies involving the immune response [5, 6].

Human tissue kallikrein-related peptidases (KLKs) constitute a subgroup of 15 secreted trypsin- or chymotrypsin-like serine proteases [7]. Their aberrant regulation has been linked to diseases such as neurodegeneration, inflammatory skin conditions and cancer [8]. There is also emerging evidence of KLKs participating in the initiation of systemic inflammatory responses and immune-modulated disorders via proteolytic cascades [9, 10]. KLK6, for instance, is strongly upregulated in activated immune cells in the central nervous system and is expressed by inflammatory cells in multiple sclerosis [11]. KLK7 can promote inflammation by cleaving pro-IL1b and producing active IL1b[12]. Moreover, pro-inflammatory roles of KLK5 and KLK6 in Netherton syndrome have been reported [13, 14]. KLK1 expression/localization in inmmune tissues/cells has also been reviewed [15]. KLK activity has also been associated with certain autoimmune diseases [16, 17].

The ability of proteolytic enzymes such as KLKs to cleave extracellular matrix (ECM) proteins has been shown to influence the migration of immune cells through tissues and vascular walls [1, 18]. KLKs also assist in innate immunity through proteolytic cleavage, which generates antimicrobial peptides from protein precursors [10, 19, 20]. KLK5, KLK6, and KLK14 cleave and activate the proteinase-activated receptor-2 (PAR2) with implications for inflammation [21]. Given the putative function of KLKs in the immune response, a better understanding of their role in immune cells may

help unravel the underlying processes behind immunological disorders and lead to new therapeutic targets [1].

We sought to investigate the expression patterns of the KLK family in immune-related lymphoid tissues, with a focus on tonsil, lymph nodes and spleen. To date, research regarding KLKs in lymphoid tissues have mostly focused on the mRNA level, where preliminary studies revealed the presence of KLK mRNA in lymph nodes and Peyer's patches [22], as well as in neutrophils [23]. In the present study, we mined public databases for KLK mRNA expression in lymphoid tissues before utilizing ELISA immunoassays to measure protein concentrations of 12 KLK family members in these tissues. Finally, we used immunohistochemical (IHC) **(Table 1)** to visualize the expression patterns of 13 KLK family members at the cellular level to provide a comprehensive picture.

2. Materials and Methods

2.1 In silico analysis of KLKs mRNA expression in immune-related tissues

The pattern of KLK mRNA expression in normal bone marrow and primary and secondary lymphoid tissue was determined by analyzing data from "The Human Protein Atlas" (https://www.proteinatlas.org/). KLK1-KLK15 expression in appendix, spleen, bone marrow, lymph node, and tonsil was indicated as number of TPM (Human Protein atlas) (http://www.proteinatlas.org/) (**Table 2**). For comparative analysis, the high, moderate and lower expression levels were arbitrarily defined as: 0-0.2 (very low), 0.3-0.5 (low), 0.6-0.9 (moderate), 1.0-10 (high), >10 (very high).

2.2 Tissue extract preparation

The Research Ethics Board (REB) of Mount Sinai Hospital, Toronto, Canada, approved this study. The following human tissues were retrieved from adult individuals at autopsy: tonsil, lymph nodes,

and spleen. Tissue extracts were processed from two patient samples and examined for 12 KLKs. Tissue cytosolic extracts were prepared from snap-frozen tissues as previously described [24]. Briefly, fresh frozen human tissues were pulverized to a fine powder in 0.2 g/ml of extraction buffer. Cell suspensions were incubated on ice for 30 min, mixing every 5 min, and centrifuged at 15,000 g for 30 min at 4 °C. The clear supernatants (cytosolic extracts) were collected and stored at -80 °C until use.

2.3 ELISA assays

Sandwich-type ELISAs for 12 KLKs were performed using in-house or commercial mouse monoclonal antibodies (mAbs) for capturing and detection as follows: 1) biotinylated mAbs (monoclonal-monoclonal ELISA configuration), or 2) rabbit polyclonal antibodies followed by alkaline phosphatase goat anti-rabbit IgG (monoclonal-polyclonal ELISA configuration) (see refs in **Table 1;** [25]). Detailed description of each antibody and protocol that was used is presented in ref. section of Table 1. In brief, microtiter plates were coated with an anti-KLK monoclonal antibody (KLK3-KLK15 at concentration of 500 ng/100uL, diluted in 50 mM Tris buffer (pH 7.8) and incubated overnight. The following day, plates were washed three times, and each of the tissue extracts, diluted in Buffer A (60 g/L BSA, 25 ml/L normal mouse serum, 100 ml/L normal goat serum, 10 g/L bovine IgG, 0.005% (v/v) Tween-20 in 50 mM Tris, pH 7.8), was added and incubated at RT (2h). Subsequently, plates were washed three times and 100 µl of each secondary biotinylated monoclonal antibody, diluted appropriately in Buffer A (KLK3 (1/2000); KLK5 (1/2000); KLK6 (1/2000); KLK7 (1/1000); KLK8 (1/1000); KLK9 (1/1000); KLK10 (1/500); KLK11 (1/1000) and KLK13 (1/500)) was added. The rabbit polyclonal antibodies were also prepared by dilutions in Buffer A (KLK4 (1/2000); KLK14 (1/2000); KLK15 (1/3000)), and incubated for 1h at room temperature (RT). Plates were washed three times. Either alkaline phosphatase-conjugated streptavidin (SA-ALP), prepared in

6% BSA (mono-monoclonal combination), or alkaline phosphatase-conjugated goat anti-rabbit IgG (mono-polyclonal combination), prepared in Buffer B (60 g/L BSA, 25 ml/L normal mouse serum, 100 ml/L normal goat serum, 10 g/L bovine IgG, 0.005% (v/v) Tween-20 in 50 mM Tris, pH 7.8, 0.5 M KCl), was added to each well, and incubated for 15 or 30 min, respectively. Then, the plates were washed six times and 100 ul of diflunisal phosphate (DFP) solution, prepared in substrate buffer (0.1 M NaCl, 1 mM $MgCl_2$ in 0.1 M Tris, pH 9.1), was added to the plate and incubated for 10 minutes at RT with gentle shaking. Subsequently, the developing solution (1 M Tris, 0.4 M NaOH, 2 mM $TbCl_3$ and 3 mM EDTA)(100 µl per well) was added and mixed for 1 min. Time-resolved fluorescence was measured with the Wallac EnVision 2103 Multilabel Reader (Perkin Elmer), as previously described [24].

2.4 Immunohistochemistry

Representative samples from three normal tonsils and two normal lymph nodes were studied by IHC. Negative controls in the tonsil and lymph node were also used, where the tissue was processed and stainined according the same procedure described below with the only difference being no primary antibodies were added. Formalin-fixed, paraffin-embedded tissues were cut to 4-um sections and stained using in-house mouse monoclonal and rabbit polyclonal antibodies, as previously described (**Table 1**). The specificity of each antibody was evaluated prior using Western blot (**Table 1**). The staining procedures involved paraffin removal with three changes of xylene (3 min each), followed by one change of xylene:/ethanol (1:1) (3 min each), and further rehydration through decreasing concentrations of ethanol (100%-95%-70%-50%) for 3 min each. Finally, the slides were rehydrated with water for 5 min. Antigen retrieval was carried out by incubating the slides in buffer (citrate; pH 6.0, 0.05 % Tween-20) in a microwave for 5 min. Following one wash with water (10 min) and two

washes with phosphate-buffered saline (PBS) (5 min each), the endogenous peroxidase activity was blocked with BLOXALL™ Blocking Solution (SP-6000, Vector Laboratories, Inc., CA, USA) for 10 min. After one wash with PBS (5 min), slides were blocked with buffer (3% BSA+2% normal goat serum) for 20 min, and each KLK primary antibody, diluted in 2% BSA, was incubated overnight at 4 ^{o}C (**Table 1**). After three washes with PBS (5 min each), the sections were then incubated with ImmPRESS peroxidase reagent anti-mouse IgG and anti-rabbit IgG (for mouse monoclonal and rabbit polyclonal primary antibodies, respectively) (ImmPress HRP reagent kit, Vector Laboratories, Burlingame, CA, USA) for 1h at RT. After three washes with PBS (5 min per), immunostaining was visualized using DAB solution (DAB kit SK-4100) for 2-10 min (brown color). Following wash with water and three washes with PBS (2 min per), sections were counterstained with hematoxylin for 1 min, washed with water (10 min), dehydrated through increasing alcohol concentrations, cleared in xylene, and finally mounted. The staining pattern, distribution of the immunostaining in each tissue, and intensity of the staining were evaluated in detail using a LEICA DMLB microscope.

3. Results

3.1 KLK mRNA expression in lymphoid tissues

KLK mRNA levels, as number of transcripts per million (TPM), in normal bone marrow and primary and secondary lymphoid tissues were investigated by mining The Human Protein Atlas database (https://www.proteinatlas.org/) (**Table 2**). Strongest expression was observed in the tonsil, with higher KLK mRNA levels for the majority of the KLK family members (**Table 2**). Specifically, KLK6, KLK7, KLK10, KLK11, KLK12 and KLK13 demonstrated relatively very high KLK mRNA levels (>10 TPM) in tonsil. KLK1, KLK4, KLK8, and KLK9 showed moderate to high levels (within 0.6-10 TPM), while KLK2, KLK3, KLK5, KLK14, and KLK15 had undetectable to low levels in tonsil

(within 0-0.5 TPM). High to very high mRNA levels of KLK2, KLK6 and KLK7 were seen in spleen (within 1-10 TPM). KLK1, KLK2, KLK3, and KLK4 were elevated in appendix (within 1-10 TPM). In bone marrow, moderate to high mRNA levels were detected for KLK1, KLK2, KLK4, and KLK10 (within 0.6-10TPM), while the other KLKs had undetectable to low levels. In lymph nodes, undetectable to low mRNA levels were seen for all KLK members, except KLK1, KLK2, KLK3, KLK4, and KLK14 (**Table 2**).

3.2 Protein levels of the KLK family in immune-related tissues

We assessed the protein concentration of KLKs in tissue extracts from the tonsil, lymph node, and spleen for the 12 family members where reliable ELISA immunoassays were available. In general, KLK levels were higher in the tonsil than the rest of the tissues analyzed, where KLK7, KLK9, KLK10, and KLK13 were detected at relatively high levels (ranging from 3.30 to 9.28 ng/mL) (**Table 3**). Moderate protein levels of KLK6 and KLK11 was observed in tonsil (2.05 and 1.81 ng/ml respectively). KLK13 also showed the highest protein level in the spleen (1.51±0.06 ng/mL), compared to other KLKs analyzed (**Table 3**).

3.3 IHC analysis of KLKs in normal tonsil and lymph nodes

IHC analysis was performed for 13 KLK family members in normal tonsil and lymph node tissues from three and two individuals, respectively. In tonsil, only KLK9, KLK10 and KLK15 showed positive staining (**Figure 1A-C**), with KLK9 and KLK10 displaying different staining patterns in tonsil (**Figure 2**). KLK9 expression was strong in the activated B-cells of the germinal center (GC) in the lymphoid follicles (**Figure 2A and B**), whereas KLK10 expression was localized to the follicular dendritic cells (FDCs) (**Figure 2C and D**). In addition, KLK15 demonstrated some staining in the

interdigitating cells in and subjacent to the tonsillar epithelium (**Figure 2E**). All other KLKs showed equivocal or undetectable staining patterns **(Supplementary Figure 1**).

In lymph node, positive protein staining was identified for KLK5 **(Figure 3A**) and KLK10 **(Figure 3B)**. KLK5 showed the strongest staining in the peripheral cells surrounding the follicles **(Figure 3A**). KLK10 showed the strongest staining in the FDCs of the lymph node tissue (**Figure 3B**), although expression in lymph node was overall weaker than in tonsil. All other KLKs were undetectable **(Supplementary Figure 2**).

4. Discussion

Aberrant KLK activity and regulation have been implicated in inflammatory diseases [11, 26], where these enzymes regulate inflammation via PARs [27] and play a role in the innate immunity via the complement cascade through interaction with C3 [28]. KLKs also digest ECM components to promote infiltration of macrophages and T-cells [10]. We sought to explore the (co)expression of KLKs in lymphoid organs to better understanding their contribution to immunity.

We first mined the Human Protein Atlas to study KLKs expression in immune-related tissues, finding that KLK mRNA levels were generally higher in the tonsil (**Table 2**). We next performed a quantitative study of KLK protein expression (KLK3-KLK15, except KLK12) by ELISA (**Table 3**), focusing on three immune-related tissues that according to mRNA expression data demonstrated: 1) high expression (tonsil), 2) moderate expression (spleen) and 3) low expression (lymph node) for most of the members of the KLK family (**Table 2**).

The significantly lower levels of KLKs in lymph node and spleen suggest that KLKs may contribute to the immune response through functions in the tonsil, but not in other lymphoid tissues

(**Table 3**). KLKs in tonsil **(Table 3)** may play roles in lymphocyte activation and cell turnover through enzymatic interactions.

The IHC data allowed us to localize KLK expression in different immune cell types. We have shown that KLK9 and KLK10 are expressed in different immune-related cells in the tonsil (**Figure 1-2**). KLK9 is strongly expressed in the GC of the lymphoid follicles, where activated B-cells are undergoing affinity maturation and class switch into either mature memory B cells or antibody-producing plasma cells. On the other hand, KLK10 is expressed in FDCs, which collaborate with B cells to ensure efficient GC formation and the production of high-affinity antibodies (**Figure 2**). It is possible that these two KLKs participate in a common cascade, similar to other members such as KLK2, KLK3, KLK5 in seminal plasma: [29], and KLK5, KLK7, KLK14 in skin [30-32]. The potential co-expression and co-regulation of KLK9 and KLK10 in a cascade may be supported by the observation that they reside adjacently on the same chromosomal locus [33]. In a canine model, the two KLKs were found to be concurrently expressed in almost all tissue samples [34]. Moreover, the association between KLK9 and KLK10 in regulating the immune response has been previously implied through their role in enzymatic cascades in sweat [35], as well as by their co-upregulation upon LPS induction via CD14 and TLR4 receptors [3]. Our previous degradomic analysis also affirmed that KLK9 cleaves KLK10 [36]. It is thus possible that the two proteases may interact in the tonsil, where activated B cells (in which KLK9 is expressed) that participate in a GC reaction can interact with surface antigens on FDCs (in which KLK10 is expressed). In the future, further IHC experiments that look at the co-localization of specific cell markers with KLKs will be necessary to further evaluate our results. Moreover, further evidence is warranted to assess the putative notion that KLK9 and KLK10 partake in the same proteolytic pathway, as well as how they may be involved in the process of B cell maturation in the tonsil.

The clinical utility of studying KLKs in immunity is also highly important for understanding anti-tumor responses in cancer. Some of the strongest evidence linking anti-tumor immunity and cancer are seen in ovarian cancer [37], where KLK9 and KLK10 have been implicated to aid in anti-tumor [38]. Similarly, KLK15 was expressed in tonsil in our study (**Figure 2E**) and may hold clinical relevance in ovarian cancer [38]. KLK10 has the highest protein expression in tonsil among other KLK members (**Table 3**) and is located in the most potent antigen-presenting cells, the FDCs [39] in both tonsil (**Figure 2C and D**) and lymph node tissue (**Figure 3B**). Other studies have previously demonstrated the integral role of KLK10 in T-cell responses to viral antigens and the development of pro-inflammatory responses, as mediated by the adaptive immune system [40]. Recent immunopeptidome analyses demonstrated that KLK10 protein is amongst the presented immunogenic epithelial ovarian cancer antigens and is a target for antigen-specific immunotherapy [41].

Overall, our study revealed the possible implications of KLK expression and regulation in immune cells. Our data support the hypothesis that a panel of KLKs have functional significance in the immune response in tonsil. With further research into the mechanisms behind their functions in immune-related tissues and their contributions to pathology, KLKs may represent promising therapeutic targets for immune-related diseases and for cancer immunotherapy.

5. Author contributions

All authors have accepted responsibility for the content of this manuscript and approved the submission.

6. Research funding

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7. Declaration of interest

The authors have no competing conflicts of interest with the contents of this article.

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Glossary

KLK kallikrein

PBS phosphate-buffered saline

IHC immunohistochemistry

BSA bovine serum albumin

ELISA enzyme-linked immunosorbent assay

ECM extracellular matrix

FDCs follicular dendritic cells

GC germinal center

SA-ALP alkaline phosphatase-conjugated streptavidin

DFP diflunisal phosphate

RT room temperature.

Figure legends

Figure 1. KLK9, KLK10, and KLK15 are expressed in normal tonsil tissue.

IHC analysis of KLKs in normal tonsil tissue showed positive protein staining for KLK9 **(A)**, KLK10 **(B)** and KLK15 **(C)** (black arrows). Negative control of tonsil **(D)** is shown where no primary antibodies were added. Magnification: 50X.

Figure 2. Localization of KLK9, KLK10 and KLK15 expression in different cell types in tonsil.

Images of immunohistochemistry staining with higher magnification (as indicated for each panel) for KLK9, KLK10, and KLK15 showed discrete patterns of cellular localization in the tonsil. KLK9 showed weak staining in the squamous epithelium (blue arrow), but positive staining in the lymphoid follicles (black arrow) **(A)**. A closer look confirms that KLK9 shows weak staining in the squamous epithelium (blue arrow), is negative in the marginal zone surrounding the lymphoid follicles (red arrow) and is strongly positive in the germinal center (GC) where activated B cells reside (black arrow) **(B)**. KLK10 demonstrated positive staining in the follicular dendritic cells surrounding the GC (black arrow) **(C-D)**, but not within the follicles (blue arrow) **(C)**. KLK15 showed weak staining in the GC (blue arrow) and positive staining in the interdigitating cells (black arrow) in and subjacent to the tonsillar epithelium **(E).** Negative control in tonsil **(F)** is shown where no primary antibodies were added.

Figure 3. KLK5 and KLK10 are expressed in different cells in normal lymph node tissue.

Immunohistochemistry analysis of KLKs showed positive staining for KLK5 **(A)** and KLK10 **(B)** in normal lymph node tissue. High magnification (as indicated in each panel) showed distinct cellular localization of KLK5 and KLK10 in the lymph node **(A-B)**. KLK5 showed strongest staining in the

peripheral cells surrounding the follicles (black arrow) **(A)**, while KLK10 showed the strongest staining in the follicular dendritic cells (black arrow) **(B)**. Negative control of lymph node **(C)** is shown where no primary antibodies were added.

463 **Tables**

464 **Table 1. Antibodies used for immunohistochemistry (IHC)[1]**

Kallikrein	Antibody type	Antibody titration for IHC	Reference
KLK3	monoclonal	1/50 to 1/100	Commercial
KLK4	polyclonal	1/200	Obiezu et al 2005[42]
KLK5	monoclonal	1/50	Yousef et al 2003[43]
KLK6	monoclonal	1/100	Diamandis et al 2000[44]
KLK7	polyclonal	1/200	Walker et al 2014[45]
KLK8	polyclonal	1/200	Kishi et al 2003 [46]
KLK9	monoclonal	1/100 to 1/200	Filippou et al 2017a[24]
KLK10	monoclonal	1/100	Luo et al 2001 [47]
KLK11	polyclonal	1/250	Diamandis et al 2002[48]
KLK12	polyclonal	1/100 to1/200	Memari et al 2007[2][49]
KLK13	polyclonal	1/200	Kapadia et al 2003 [50]
KLK14	monoclonal	1/50 to 1/200	Borgono et al 2007 [30]
KLK15	monoclonal	1/250	Filippou et al 2018 [51]

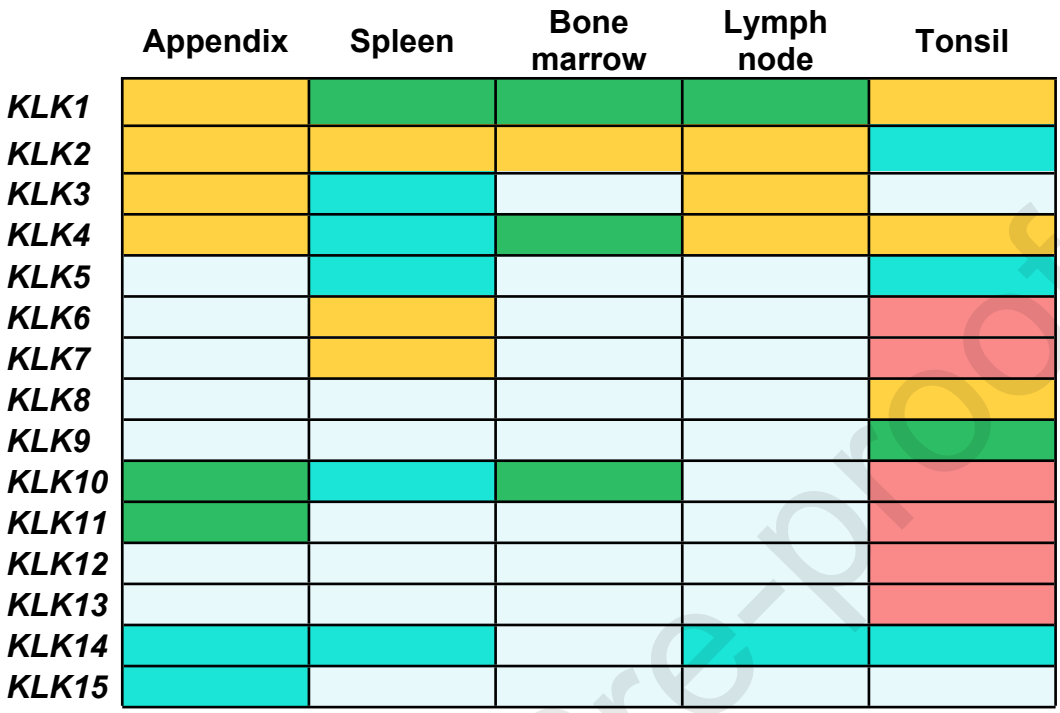
465

466 [1]All monoclonal antibodies were raised in mice; all polyclonals in rabbits. All monoclonals had a
467 concentration of 1 mg/mL.
468
469 [2]KLK12 antibody was only used for IHC analysis. ELISA immunoassay was not performed for KLK12
470 in immune-related tissues for this study.
471

472

473 **Table 2. mRNA levels of KLKs in normal immune-related tissues from the Human Protein**

474 **Atlas[1] database**



0-0.2	0.3-0.5	0.6-0.9	1.0-10	> 10	**Transcripts Per Million (TPM)**
Very low	Low	Moderate	High	Very high	**mRNA level**

475

476 [1]www.proteinatlas.org

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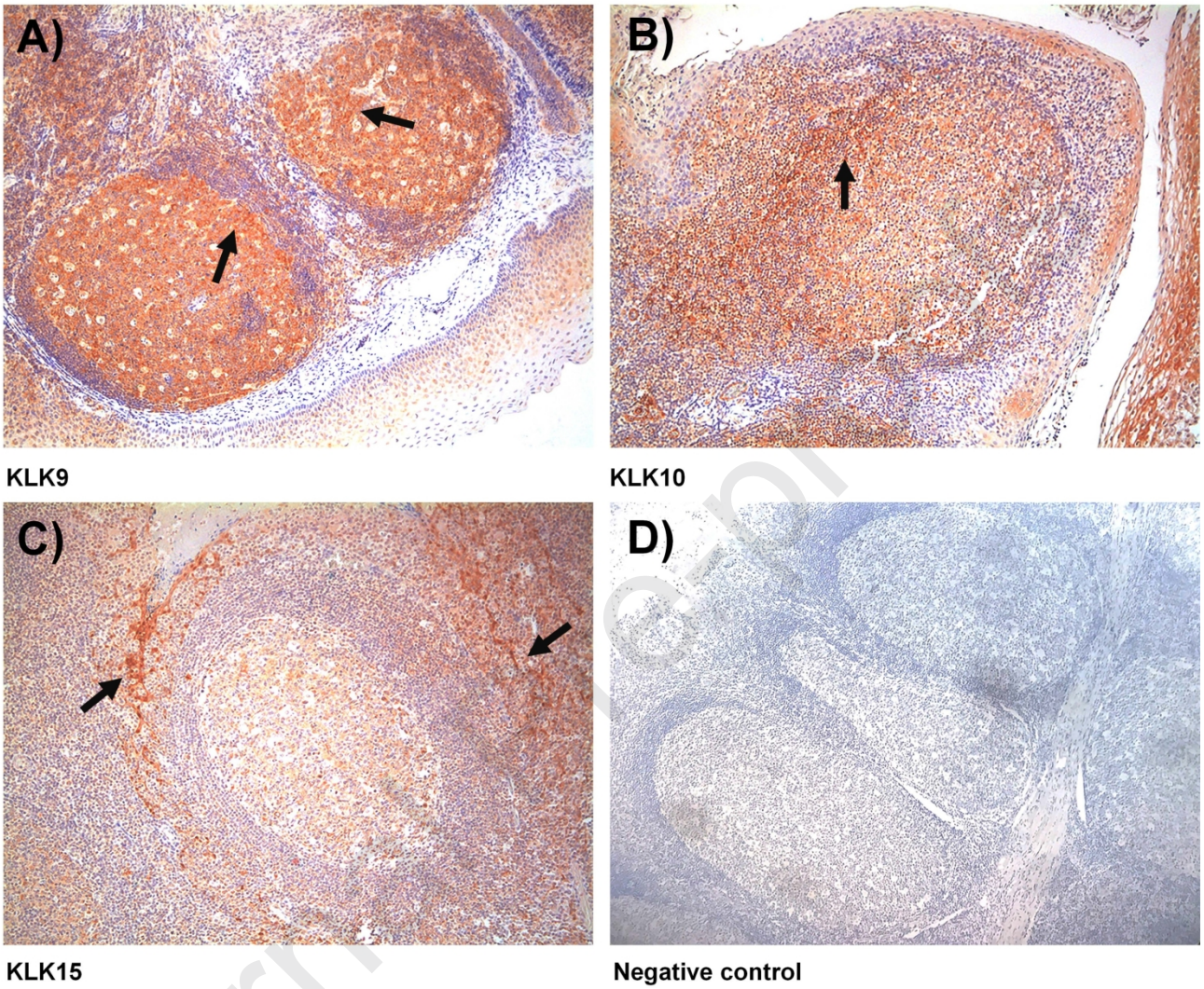
484 **Table 3. KLK protein concentration in cytosolic extracts quantified by ELISA[1]**

KLKs	Tonsil (ng/mL)	Lymph node (ng/mL)	Spleen (ng/mL)
KLK3	ND[2]	1.35(±0.02)	ND
KLK4	0.40 (±0.04)	ND	ND
KLK5	1.20 (±0.007)	0.24 (±0.004)	0.11 (±0.0001)
KLK6	2.05 (±0.04)	0.13 (±0.004)	ND
KLK7	3.30 (±0.06)	0.9 (±0.02)	0.085 (±0.01)
KLK8	0.39 (±0.007)	0.02 (±0.0005)	ND
KLK9	4.0 (±0.04)	0.2 (±0.01)	0.02 (±0)
KLK10	9.28 (±0.07)	0.11 (±0)	0.14 (±0.0075)
KLK11	1.81 (±0.04)	0.2 (±0.01)	0.06 (±0)
KLK13	5.18 (±0.34)	0.19 (±0.02)	1.51 (±0.06)
KLK14	0.23 (±0.012)	0.05 (±0.004)	ND
KLK15	0.6 (±0.007)	0.28 (±0.006)	0.2 (±0.01)

485

486 [1]All extracts were adjusted to 1 mg/mL of total protein. Values are means ± standard deviation of
487 triplicates
488 [2]ND, not detected

489 **Figures**

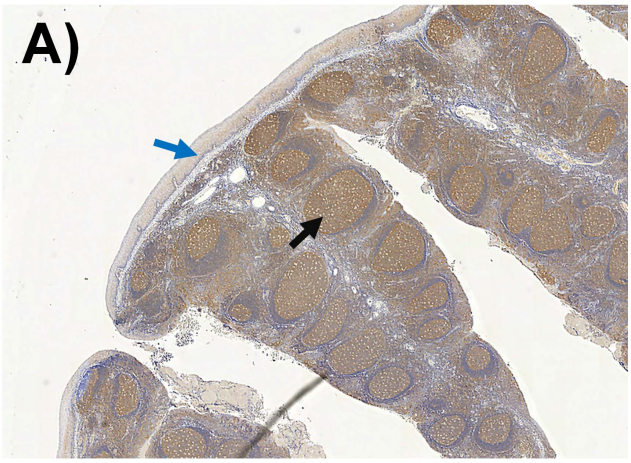


490

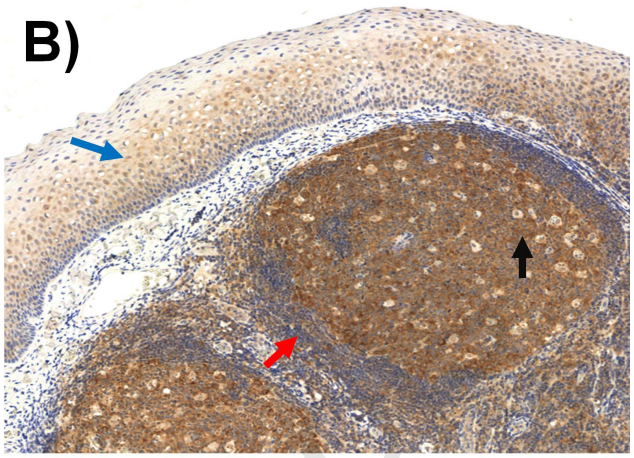
491

492 **Figure 1**

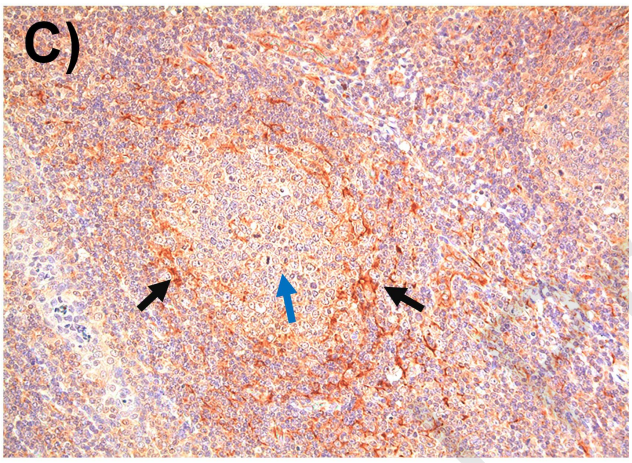
493



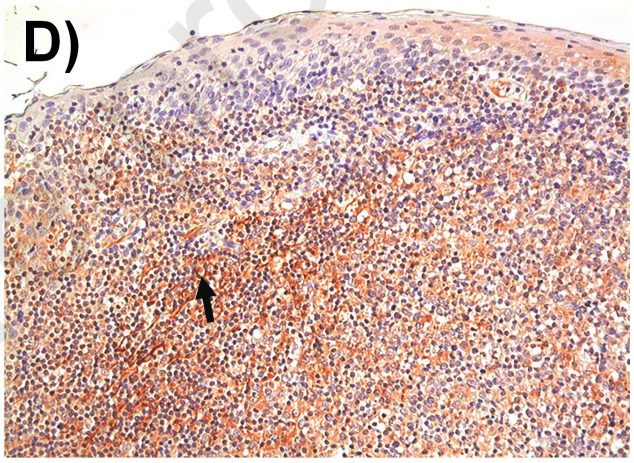
KLK9 (25X)



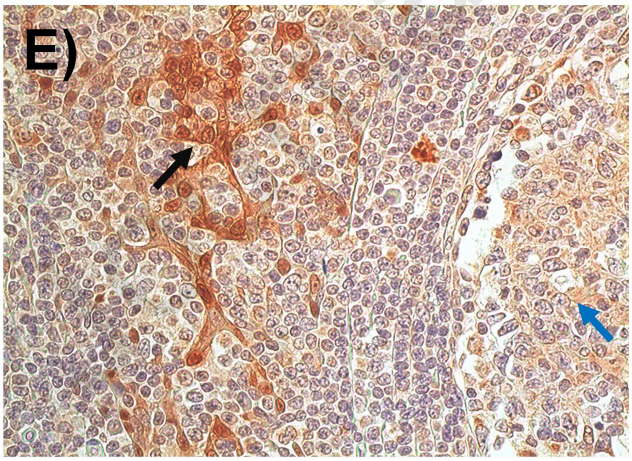
KLK9 (100X)



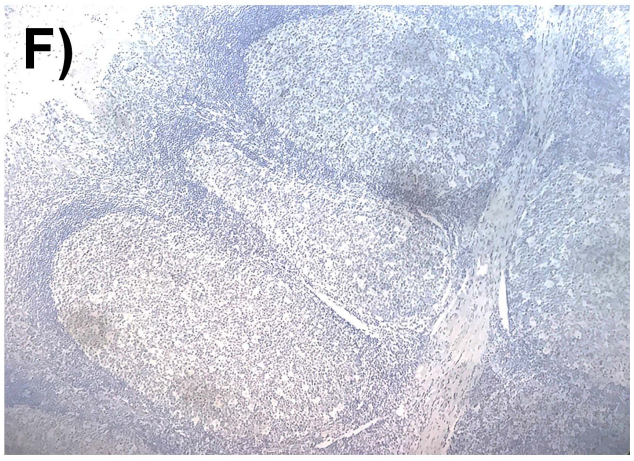
KLK10 (100X)



KLK10 (200X)



KLK15 (400X)



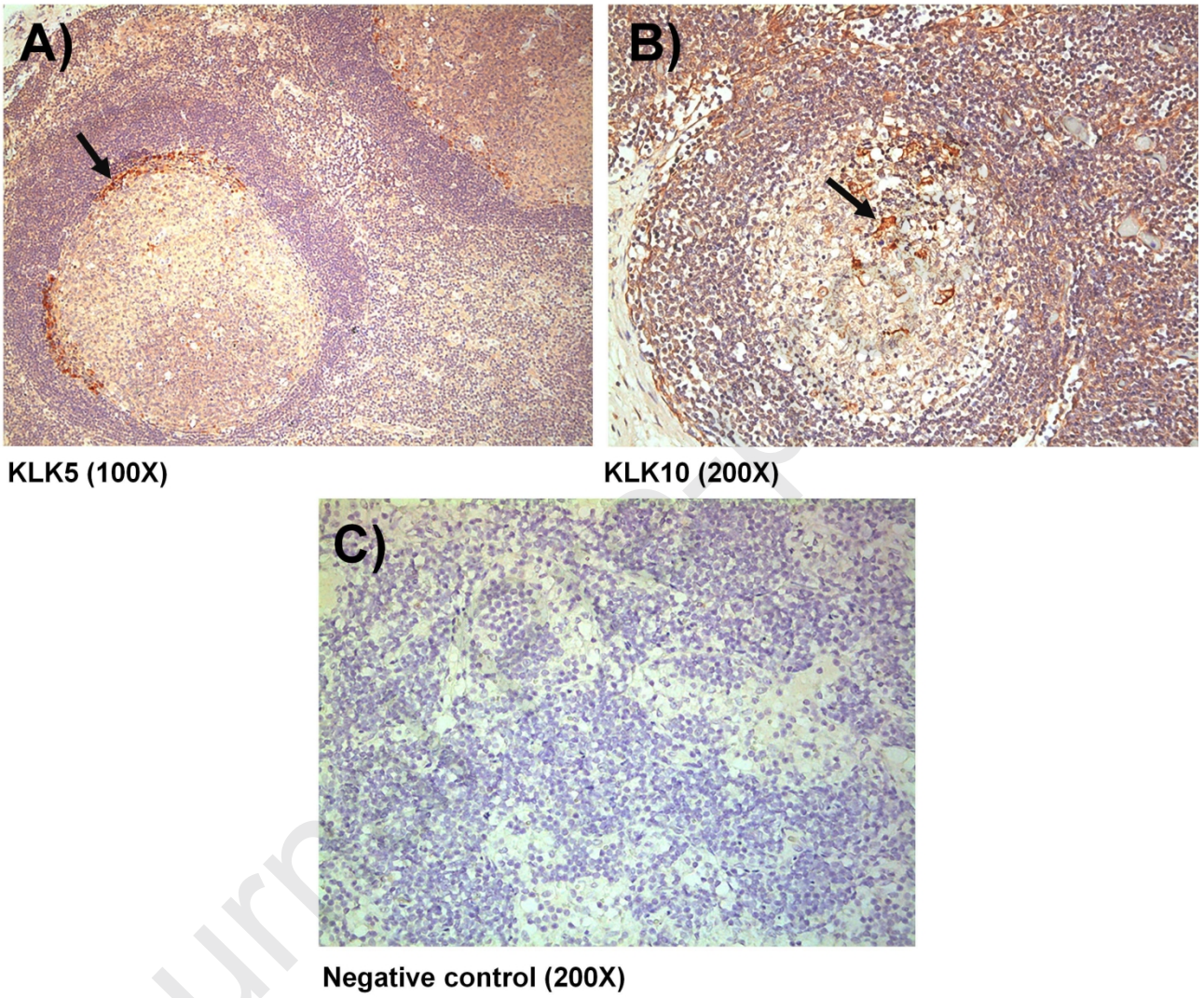
Negative control (50X)

494

495 **Figure 2**

496

497



498

499 **Figure 3**

500